



Evaluation of *Optineurin* as a candidate gene in Indian patients with primary open angle glaucoma

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Purpose: To evaluate the role of the *optineurin* gene (*OPTN*) in Indian primary open angle glaucoma (POAG) patients from different parts of the country.

Methods: Two hundred patients with POAG and 200 ethnically matched normal controls were recruited from various parts of India for the study. The entire coding region of *OPTN* along with the intron-exon boundaries were screened by PCR and single strand conformation polymorphism (SSCP) followed by direct sequencing. A rapid screening method was developed for some of the observed variants by denaturing high performance liquid chromatography (dHPLC). Four variants were also confirmed by digesting the amplicon with appropriate restriction enzymes.

Results: Seven nucleotide changes were observed in *OPTN* of which one was a putative mutation in exon 16 (Arg545Gln) that was observed in six POAG patients and not in the controls ($p < 0.05$). The remaining variants comprised four single nucleotide polymorphisms (SNPs) in the coding region (Thr34Thr, Met98Lys, Arg149Arg, and Asn303Lys) and two in intron 6 (879-10G>A and 879-5C>T). But frequencies of the minor allele were not significantly different among the patients and controls. The Met98Lys variant that was identified to be a potential risk factor for NTG and POAG in some Asian populations and also for modulating IOP in Caucasian populations, did not exhibit any significant association to the disease phenotype.

Conclusions: Despite a putative mutation (Arg545Gln) in some patients, the present study does not suggest a significant involvement of *OPTN* in POAG patients of Indian origin.

The glaucomas comprise a group of heterogeneous optic neuropathies with a complex genetic basis. If untreated, these neuropathies lead to optic nerve damage along with a progressive loss of vision [1,2]. According to latest estimates, glaucoma affects about 67 million people worldwide [3] and 1.5 million in India [4]. Among the various glaucoma subtypes, primary open angle glaucoma (POAG) is more common and accounts for half of these cases [5]. Seven chromosomal loci (*GLC1A-GLC1G*) have so far been implicated in POAG [6-12], of which three genes, *myocilin* (*MYOC*) on *GLC1A* (1q32) [13], *optineurin* (*OPTN*) [14] on *GLC1E* (10p25), and *WDR-36* on *GLC1G* (5q22.3) [12] have been characterized. In addition, a few other chromosomal loci have been mapped for POAG through genome-wide scans on 2q,

9q, 10p, 14q, 15q, and 22p that do not overlap with the previously implicated loci [15-17]. Through bioinformatic analysis, the Noelin family of genes has also been suggested to be involved in ocular pathogenesis leading to glaucoma [18].

Among the three glaucoma genes, the potential role of *MYOC* has been well documented in juvenile and adult-onset POAG [19]. Globally, *MYOC* mutations account for 2-5% of all POAG cases [1,2,19]. On the other hand, defects in *OPTN* have been implicated in normal tension glaucoma (NTG) and mild to moderate forms of late-onset POAG [14,20]. However, mutations in both *MYOC* and *OPTN* genes could have considerable overlaps in phenotypic expressions.

An initial report on 52 POAG families demonstrated the involvement of *OPTN* mutations in 16.7% along with an additional risk to 13.6% of familial and sporadic cases due to the intragenic SNPs [14]. Following this discovery, a large number of studies were undertaken to identify the defects in *OPTN* causal to NTG and POAG [21-31]. One such study screened 1,048 patients for variations previously associated with glaucoma [21]. Among the previously reported mutations [14], they detected one individual with familial NTG and British ancestry to have the Glu50Lys mutation. The Arg545Gln was described as a polymorphism that does not cause disease

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and the previously reported frame-shift mutation was not detected in any of their patients. Additionally, there are contradicting reports from different ethnic groups particularly from Asia that investigated the involvement of *OPTN* in POAG and/or NTG [27-30], but none from India. The present study was therefore aimed at understanding the possible role of *OPTN* in the causation of POAG among Indian patients from different parts of the country.

METHODS

Selection of cases: The study protocols adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board. Two hundred Indian patients (mean age 48.7 ± 16.8) with or without a family history of POAG presenting at the LV Prasad Eye Institute, Hyderabad, Eye Care & Research Center, and Regional Institute of Ophthalmology, Kolkata, were recruited. All the POAG cases had an elevated intraocular pressure of >21 mm Hg and/or glaucomatous disc changes in the presence of typical field defects, along with an open angle on gonioscopy and no other secondary causes. Cases with a history of inflammation, ocular trauma, ocular hypertension, and normal tension glaucoma were excluded [32]. Two hundred ethnically matched normal individuals (mean age 56.2 ± 12.1) without any signs or symptoms of glaucoma or other associated ocular or systemic diseases served as controls.

Collection of samples and DNA analysis: Blood samples were collected in EDTA by venipuncture with prior informed consent from the POAG patients and controls. Genomic DNA was prepared using the conventional phenol-chloroform extraction method. The coding region of *OPTN* (NM_021980; contig containing *OPTN*: NT_077569) was amplified by polymerase chain reaction (PCR) using exon-specific primers (designed and kindly provided by Dr. Mansoor Sarfarazi, University of Connecticut, Farmington, CT). PCR was carried out in a total volume of 25.0 μ l containing 50-100 ng genomic DNA, 0.4 μ M of each primer, 0.2 mM of each dNTP, appropriate

concentration of $MgCl_2$, and 0.5 unit of Taq polymerase (Invitrogen, Carlsbad, CA) in a GeneAmp-9700 thermocycler (PE Applied Biosystem, Foster City, CA).

Mutation screening by SSCP and dHPLC: Mutation screening was done by single-strand conformation polymorphism (SSCP) of the PCR amplicons in 8% non-denaturing polyacrylamide gels run at different temperatures with and without glycerol. Prior to loading, amplicons were denatured at 95 °C for 5 min and snap chilled on ice to prevent reannealing of the single stranded product. Electrophoresis was performed at a constant 160 volts for 18-22 h, depending on the sizes of the amplicon. Following electrophoresis the gels were silver stained to visualize the bands. Samples exhibiting band shifts were further analyzed by direct sequencing.

The SSCP variants were screened by denaturing high-performance liquid chromatography (dHPLC) in the Wave Analyzer (Transgenomic Inc., Omaha, NB) for high-throughput screening in additional patient and control samples. Prior to loading, the samples were denatured at 96 °C for 5 min and were gradually cooled down to 65 °C to facilitate heteroduplex formation. The melting temperatures were calculated from the WAVEMAKER™ software (version 4.1) and were based on fragment length, sequence, and the position of the variant in the amplicon. Samples were run under partially denaturing conditions and the elution of heteroduplex peaks was monitored from the chromatogram.

DNA sequencing: The variants observed in SSCP/dHPLC were characterized through bi-directional sequencing of the amplicon. The PCR amplicons were column-purified using the Qiagen PCR-purification kit (Qiagen, Hilden, Germany), and sequencing was performed on an ABI Prism 3100 DNA sequencer using the BigDye chemistry. Nucleotide changes were detected from the chromatogram by comparing with the normal *OPTN* gene sequence available in the database by Pairwise BLAST [33].

Restriction enzyme digestion: Nucleotide variants identified by DNA sequencing, that altered restriction sites, were

TABLE 1. NUCLEOTIDE CHANGES IN THE *OPTN* GENE IN INDIAN POAG PATIENTS

| Nucleotide change | Position in gene | Mutation/SNP | Restriction site (+/-) | Amino acid change | Observed genotypes (wt, wt/wt, mt/mt, mt) | |
|-------------------|------------------|-----------------|------------------------|-------------------|---|------------|
| | | | | | Patients | Controls |
| c.428 G>A | Exon 4 | SNP (rs2234968) | HpyCh4IV (-) | Thr34Thr | 134/61/ 5 | 106/83/11 |
| c.619 T>A | Exon 5 | SNP | StuI (+) | Met98Lys | 178/22/ 0 | 189/11/ 0 |
| c.879-10 G>A | Intron 6 | SNP | | | 197/ 3/ 0 | 100/ 0/ 0* |
| c.879-5 C>T | Intron 6 | SNP (rs2244380) | | | 12/55/133 | 6/28/66* |
| c.1960 G>A | Exon 16 | Mutation | Acil (-) | Arg545Gln | 194/ 6/ 0 | 200/ 0/ 0 |
| c.773 G>A | Exon 6 | Novel SNP | | Arg149Arg | 99/ 0/ 1 | 99/ 1/ 0* |
| c.1054 C>A | Exon 10 | Novel SNP | MnlI (-) | Asn303Lys | 199/ 1/ 0 | 194/ 6/ 0 |

Nucleotide changes in the *OPTN* gene in Indian POAG patients are essentially nonpathogenic. In the "Restriction site (+/-)" column, the sign indicates the creation or abolition of a restriction site for *HpyCh4IV* (*HpyCh4IV*), *StuI* (*StuI*), *Acil* (*Acil*), or *MnlI* (*MnlI*). The terms "wt" and "mt" indicate the wild type allele and the mutant allele, respectively. The asterisks indicate that these intronic and synonymous variants were screened in 100 normal controls. Observed genotypes were scored to estimate the potential association of specific nucleotide variants with the phenotypes (i.e., POAG or normal). While no significant variation was observed for any SNP, the reported mutation (Arg545Gln) was detected only in six heterozygous patients, but not in the controls.

screened in additional POAG patients and control samples by digesting the amplicon with appropriate restriction enzyme (Table 1) as per the manufacturer's protocol (New England BioLabs, Beverly, MA). DNA fragments in the digest were separated by electrophoresis in 6% polyacrylamide gels, stained with ethidium bromide, and visualized under an UV transilluminator. Alleles were scored based on the band patterns in the gel.

RESULTS & DISCUSSION

Screening of *OPTN* in a pool of 200 unrelated POAG patients of Indian origin revealed seven different nucleotide variants (Table 1) of which only the Arg545Gln was a potential mutation that was originally reported by Rezaie et al. [14], in 2002. Among the other variants, the Met98Lys was previously reported to be a risk factor in certain populations [22,24] and the remaining comprised of five innocuous single nucleotide changes. Our results emphasize a limited involvement of *OPTN* in Indian POAG patients similar to most other studies [23,26,30,31].

Arg545Gln as a potential mutation: We detected a nucleotide variant c.1960G>A in 6/200 POAG patients (3.0%) that represented a missense mutation Arg545Gln in exon 16 in the present study. This variant creates a restriction site for *Acil* and was absent in 400 normal chromosomes. Interestingly, all of these patients (6) were from eastern India, and two of them

had a juvenile onset of the disease symptoms. Haplotypes generated through intragenic single nucleotide polymorphisms (SNPs) indicated multiple independent origins for the Arg545Gln variation (data not shown). Although Arg545Gln does not belong to any known protein domain of *OPTN*, it is located near the zinc finger motif, the latter being normally seen in transcription factors.

This variant was initially identified only in a patient and was absent in normal controls that led investigators to term it as a mutation [14]. Since then there has been multiple reports on various ethnic groups contradicting the status of this variant as a pathogenic mutation (Table 2). This mutation has not been observed in any other patient of Caucasian origin from Canada, the United States, Germany, and Australia [21,25,26,31]. As evident from Table 2, the Arg545Gln mutation has been observed predominantly in Asian populations, with varying frequencies. A large series of 1,048 patients from the United States [21] and 66 patients from Canada [25] observed the Arg545Gln only in 12 NTG patients (3.4%) of Japanese and one JOAG patient of Chinese origins, respectively. These variants were also observed in Japanese and Chinese control chromosomes, although the differences were not statistically significant. Similarly another study on 119 POAG subjects from China observed this variant in almost equal frequencies among the patients (6.7%) and controls (8.7%) [27].

A recent report on Japanese patients observed the Arg545Gln change only in one NTG patient and not in the POAG cases or controls [30], similar to the initial report of Rezaie et al. [14]. However, two large studies from Japan re-

TABLE 2. DISTRIBUTION OF THE ARG545GLN VARIANT

| Population | Variants present (%, total) | | References |
|------------|--------------------------------|------------|---------------|
| | Patients | Controls | |
| Australia | 0.00 (27) | 0.00 (94) | [26] |
| Canada | 1.51 (66) | 1.63 (184) | [25] |
| Germany | 0.00 (112) | 0.00 (100) | [31] |
| UK | 0.00 (315) | 0.00 (95) | [22] |
| USA | 0.00 (86) | 0.00 (80) | [23] |
| USA | 0.09 (1048) | 1.19 (251) | [21] |
| USA | 2.17 (46) | 0.00 (100) | [14] |
| China | 6.72 (119) | 8.73 (126) | [27] |
| Japan | 0.06 (154) | 0.00 (100) | [30] |
| Japan | 6.32 (411) | 5.04 (218) | [29] |
| Japan | 6.38 (313) | 5.10 (196) | [28] |
| India | 3.00 (200) | 0.00 (200) | Present study |

Variable distribution of Arg545Gln in open angle glaucoma (OAG) patients and controls in different population groups of the world. Among the population groups from consisting mostly of Caucasians a single study [14] reported the presence of the variant allele, coding for 545Gln, among the OAG patients. However, the frequencies of the variant allele in the OAG patients and controls were comparable among east Asians except for one study [30] that observed this variant in an NTG patient and not in the controls. Our study on Indian POAG patients identified the variant allele only among the patients similar to the original report [14] that implicated *OPTN* in open angle glaucoma.

TABLE 3. FREQUENCY DISTRIBUTION OF THE MET98LYS VARIANT ALLELE

| Population | Patients | | Controls (%) | References |
|------------|----------|---------|--------------|---------------|
| | POAG (%) | NTG (%) | | |
| Australia | 7.4 | 0.0 | 3.2 | [26] |
| Canada | 12.2 | 0.0 | 3.9 | [25] |
| France | 4.6 | 0.0 | 4.5 | [24] |
| Germany | 0.0 | 6.3 | 7.0 | [31] |
| Morocco | 10.7 | 0.0 | 8.3 | [24] |
| UK | 4.4 | 10.6 | 3.2 | [22] |
| USA | 1.8 | 11.8 | 4.3 | [14] |
| USA | 5.1 | 5.3 | 7.2 | [21] |
| USA | 9.0 | 0.0 | 10.0 | [23] |
| China | 28.6 | 0.0 | 24.6 | [27] |
| Japan | 16.9 | 15.4 | 5.0 | [30] |
| Japan | 17.0 | 22.1 | 16.5 | [29] |
| Japan | 13.3 | 19.6 | 13.8 | [28] |
| India | 11.0 | 0.0 | 5.5 | Present study |

Frequency distribution of variant alleles coding for Met98Lys in POAG and NTG patients in different population groups of the world. The table highlights the variability in the reported studies on the relative frequency of the allele coding for 98Lys among the population groups consisting mostly of Caucasians, east Asian, and south Asian. Such variability in the allelic distribution might be due to different gene pools in the population groups which determine the interaction of *OPTN* with other proteins contributing to the pathophysiology of the disease.

ported strikingly similar frequencies of this variant in cases of POAG, NTG, and controls [28,29]. It has been claimed that the Arg545Gln mutation could largely be a polymorphism that does not cause disease in Asian populations [21,27], although the present study did not observe this variant in unrelated normal individuals. Unlike other studies, our results indicate a significant association of Arg545Gln in POAG ($p < 0.05$). Variable penetrance of the *OPTN* variant in different ethnic populations suggests potential involvement of other modifying loci, which might differ from one population to the other. Further, it highlights the importance of screening this variant in Indian POAG populations to establish its potential role in causation of POAG.

Is the Met98Lys a potential risk factor?: The nucleotide variant c.619T>A (Met98Lys) was detected both in patients and controls by restriction digestion of the PCR amplicon with *StuI* as described earlier [22] followed by confirmation of the variants by direct sequencing. The frequency of the minor allele among the patients (0.05) and controls (0.03) was statistically not significant. None of the individuals screened exhibited this variant in the homozygous condition (Table 1).

The Met98Lys variant was initially identified as an “attributable risk factor” as it was observed in significantly higher frequencies in patients (13.6%) than control chromosomes (2.1%) [14]. However, the other studies from American populations did not reveal any significant differences in the frequencies of the Met98Lys variant among the patients and controls (Table 3). The large multi-ethnic study on 1,048 patients did find a significant association of Met98Lys ($p = 0.01$) among the 247 Japanese NTG patients in their subset compared to Japanese control subjects ($n = 89$), but the sample size in this subset was relatively small to perform a case-control study [21]. Similar results were replicated from a study among Japanese patients that claimed the Met98Lys variant to be a risk factor in POAG ($p = 0.009$) and NTG ($p = 0.029$), respectively [30]. Another independent study from Japan observed a relatively higher frequency of the Met98Lys variant allele in 217 NTG (22.1%) compared to 194 POAG (17.0%) patients and controls (16.5%), although the differences were not significant ($p = 0.139$ for NTG and $p = 0.893$ for POAG) [29]. Another Japanese study did not support this conclusion [28].

Later Met98Lys was reported to be a modifier of initial IOP in POAG patients [24]. Another study involving POAG and NTG patients from London showed that Met98Lys was associated specifically with NTG but not with HTG [22]. In light of these observations, a study comprising of only NTG patients is warranted to test for any causal association among Indian patients.

Other nucleotide variants: A novel heterozygous change was identified at c.1054C>A causing a substitution of asparagine by lysine (Asn303Lys) in a sporadic POAG case from eastern India that abolished a restriction site for *MnII*. The segregation of mutant alleles could not be confirmed because DNA samples from his parents were not available. However this nonconservative change was also seen in 6 ethnically matched normal controls from eastern India. Although the wild

type residue of this variant is highly conserved across species (data not shown), it is unlikely to play a potential role in the disease pathogenesis. However in absence of a functional assay we cannot comment any further on this issue.

The remaining four nucleotide changes were either intronic or silent changes that are unlikely to be involved in the pathogenesis. A silent change at c.428G>A (Thr34Thr) was detected in exon 4 that abolishes a restriction site for *HpyCh4IV*. In our series, the frequency of the variant allele among the patients (0.18) and controls (0.26) was not significant ($p > 0.5$). This change was found in almost all the studies undertaken across different populations including the Asians [14,27-30].

We observed two intronic variants upstream of exon 7 at IVS6-10G>A and IVS6-5C>T. As these changes did not create or abolish any restriction site, the entire pool of patients and controls were sequenced for this locus and the frequencies of the variant allele were identical for patients and controls (Table 1). Since both changes were intronic, not predicted to create cryptic splice sites and two variants were already reported as SNPs [27], its role in causation of the disease could be ruled out. However, like the silent change in exon 4 (Thr34Thr), the more frequent SNP (IVS6-5C>T) could be used as an informative marker in POAG pedigrees.

The present study also did not observe significant association to any SNPs in patients and controls, similar to most other studies [21,25-28,31]. As *OPTN* is commonly associated with NTG, it seems reasonable that further studies on the involvement of *OPTN* in glaucoma should be conducted, specifically on NTG patients. While there are some studies exhibiting association of *OPTN* to POAG and NTG from different ethnic groups of Asia [27,29], its major role in the disease process leading to POAG is still debatable. Despite finding a putative mutation (Arg545Gln), the present study did not show any evidence for the involvement of *OPTN* in the pathogenesis of POAG. It is worth noting that unlike the mutation spectrum in *MYOC* causing POAG [19], there are relatively fewer mutations in *OPTN* that would lead to POAG or NTG [20]. By and large the nucleotide variants observed across populations have largely been SNPs that are infrequently associated with the disease phenotype [20-31]. It is evident that *OPTN* exhibits wide variations in different ethnic groups and may be involved in POAG and primarily with NTG through a complex biochemical pathway that merits functional studies of the *OPTN* protein.

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